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## Determination of the Base Composition of Deoxyribonucleic Acid by Measurement of the Adenine/Guanine Ratio

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A method is described for determination of the base composition (as guanine + cytosine or adenine + thymine content) of DNA by accurate measurement of the adenine/guanine ratio. The DNA is hydrolysed with 0.03N-hydrochloric acid for 40 min. to release the purines. The hydrolysate is subjected to ion-exchange chromatography on Zeo-Karb 225. Apurinic acids are eluted with 0.03N-hydrochloric acid and then guanine and adenine are eluted separately with 2N-hydrochloric acid. Guanine and adenine are each collected as a single fraction, and the amount of base in each case is determined by measuring the volume and the extinction at suitable wavelengths. For use in the calculations, millimolar extinction coefficients in 2N-hydrochloric acid of 12.09 for adenine at 262 m $\mu$ , and 10.77 for guanine at 248 m $\mu$ , were determined with authentic samples of bases. The method gives extremely reproducible results: from 12 determinations with calf thymus DNA the adenine/guanine molar ratio had a standard deviation of 0.011; this corresponds to a standard deviation in guanine + cytosine content of 0.2% guanine + cytosine.

The fact that in double-stranded DNA the number of adenine residues equals the number of thymine residues, and the number of guanine residues equals the number of cytosine (or cytosine + 5-methylcytosine) residues, means that to determine the base composition (as GC content or AT content) of the DNA it is sufficient to measure the A/G ratio. The advantage of such a procedure is that it is possible to measure the A/G ratio by techniques that avoid some of the pitfalls of methods involving direct measurement of the amounts of all four (or five) bases. For instance, liberation of all the bases from DNA requires violent acid hydrolysis, such as treatment with 70% perchloric acid at 100° for 1 hr. (Marshall & Vogel, 1951) or with anhydrous formic acid at 175° for  $\frac{1}{2}$ -2 hr. (Vischer & Chargaff, 1948). Concentrated acid at high temperature may cause some degradation of bases: Abrams (1951) reported that 1.0N-hydrochloric acid at 100° for 60 min. caused 7-8% deamination of adenine and guanine. Another disadvantage of treatment with concentrated acid (particularly with perchloric acid)

is that it may lead to the formation of charcoal from the sugar moiety of the DNA and from any contaminating polysaccharide that may be present. The charcoal can adsorb nucleic acid bases (J. Levenbook, unpublished work quoted by Wyatt, 1955), giving rise to false values for the base composition. Contamination by polysaccharide is particularly likely to be serious in DNA samples isolated from plants. Confining attention to adenine and guanine can avoid these disadvantages, since the purine bases can be liberated from DNA by very mild acid hydrolysis. Tamm, Hodes & Chargaff (1952) found that the purines were quantitatively liberated from DNA by heating at 100° for 60 min. at pH 2.8. Under mild acid conditions there is no formation of charcoal and it seems likely that there is little degradation of bases.

To take advantage of the benefits attached to estimating only the purines a method for measurement of DNA base composition was devised that involved liberation of these bases from DNA by heating at 100° for 60 min. at pH 2.74, separating adenine and guanine from the hydrolysate by ion-exchange chromatography and measuring the extinctions of all the fractions at suitable wavelengths to determine concentration (Kirk, 1967). This method was used to detect a difference in base composition between chloroplast DNA and nuclear DNA in the broad bean, and with these DNA

\* Abbreviations: GC content, proportion of guanine + cytosine (+ 5-methylcytosine, where present) in DNA, expressed as a percentage of the total number of moles of purine and pyrimidine bases present; AT content, proportion of adenine + thymine, expressed in the same way; A/G ratio, adenine/guanine molar ratio.

species it was found that the standard deviation of the values of the A/G ratio was 0.03. With broad-beam nuclear DNA, which has a GC content of 39.4%, a standard deviation of 0.03 for the A/G ratio corresponds to a standard deviation of 0.5% GC content. The reproducibility of this technique therefore compared favourably with that of other methods. However, it was thought that the maximum attainable accuracy made possible by this approach had not been achieved. The technique has therefore been refined further. The new method differs from the old in that the DNA is hydrolysed at a lower pH for a shorter time, a different ion-exchange resin and an altered elution system are used to isolate the adenine and guanine, and the bases are estimated by a substantially more accurate spectrophotometric procedure. This modified technique has made possible a marked improvement in the accuracy with which DNA base composition can be measured.

## METHODS AND MATERIALS

**Extinction measurements.** All extinction measurements were carried out with a Hitachi Perkin-Elmer 139 spectrophotometer fitted with a photomultiplier. The accuracy of the monochromator was checked with a holmium filter. A slit width of 0.1 mm. was used, which gives a spectral band-pass of 0.2 m $\mu$ . To eliminate the human error involved in reading the extinction scale, and possible deviations from linearity of the meter itself, the digital read-out jack of the spectrophotometer was connected to a Solartron model LM1420.2 digital voltmeter (Solartron Electronic Group Ltd., Farnborough, Hants.). With the appropriate blank cuvette in position the signal from the spectrophotometer, which is proportional to transmittance, was adjusted to give a reading of 1.000 V on the voltmeter. When a cuvette containing a sample was moved into the light-beam its transmittance could be read to an accuracy of 0.001. In accordance with the recommendations of the manufacturers of the spectrophotometer, the concentrations of the various samples were chosen so as to give values of transmittance between 0.200 and 0.500 (i.e. extinction values between 0.699 and 0.301) to keep errors to a minimum. Transmittance values were converted into extinctions by reference to a Table of values of extinction for all values of transmittance from 0.001 to 0.999, computed on an ILM 1620 Computer. All extinction values obtained with the 5 cm. cells were corrected for slight differences in the extinctions of these cells at certain wavelengths. No such corrections were necessary with the 1 cm. cells.

**Extinction coefficients.** In this method adenine and guanine were estimated in 2N-HCl. Extinction coefficients for these bases in 2N-HCl had to be determined since the appropriate values are apparently not available in the literature. The absolute accuracy of the Hitachi Perkin-Elmer spectrophotometer when used with the 1 cm. quartz cells was checked with a standard solution of  $K_2Cr_2O_7$  in 0.01N- $H_2SO_4$ ; the observed extinction values at 235, 257, 313 and 350 m $\mu$  differed by not more than 0.3% from the theoretical values.

(a) Adenine. The extinction values of a solution contain-

ing 5.79  $\mu$ g. of adenine/ml. in 2N-HCl were measured at a series of wavelengths. The absorption maximum was found to be at 252 m $\mu$ . Since adenine should have no significant extinction at 310 m $\mu$ , the very small extinction (0.002) at 310 m $\mu$  was subtracted from the values at other wavelengths, as a partial correction for possible light-scattering effects. The millimolar extinction coefficient at 262 m $\mu$  was found to be 12.09. The  $E_{262}/E_{252}$  ratio was 1.614.

(b) Guanine. The extinction values of a solution containing 6.47  $\mu$ g. of guanine/ml. in 2N-HCl were measured at a series of wavelengths. The absorption maximum was found to be at 243 m $\mu$ . As with adenine, extinction values were corrected by subtracting the extinction (0.004) at 310 m $\mu$ . The millimolar extinction coefficient at 245 m $\mu$  was found to be 10.77. The  $E_{245}/E_{255}$  ratio was 1.557.

**DNA hydrolysis.** Calf thymus DNA was dissolved at 0.7 mg./ml. in 15 mN-KCl. Then 1 ml. of the DNA solution was pipetted into a 10 mm. x 75 mm. Pyrex test tube, and 0.1 ml. of 0.33N-HCl, giving a final HCl concentration of 0.03N, was added. The tube was placed in a boiling-water bath for 50 sec. and then sealed tightly with a rubber bung. After a further 40 min. in the water bath the tube was removed and cooled. Increasing the hydrolysis time to 60 min. did not release any more adenine or guanine from the DNA, and consequently gave exactly the same A/G ratio as 40 min. hydrolysis. A 40 min. hydrolysis time was therefore used in all the determinations.

**Separation and estimation of adenine and guanine.** Adenine and guanine were separated from the other hydrolysis products by a modification of the method of Cohn (1955). Separation was carried out on a bed of Zeo-Karb 225 resin (17 cm. high x 1 cm. diam.) in a Gallenkamp CL-120 chromatography column (A. Gallenkamp and Co. Ltd., London, E.C. 3). The column effluent was passed through a flow cell in a Unicam SP.300 recording spectrophotometer and its extinction at 250 m $\mu$  was monitored continuously. In every run, before the DNA hydrolysate was put on the column, 0.03N-HCl was run through until the extinction of the effluent fell to zero (there being a cuvette containing pure 0.03N-HCl in the reference cell compartment). This step was necessary to remove ultra-violet-absorbing impurities that slowly leach out of the resin when it is allowed to stand for any prolonged period. At least 20 ml. of 0.03N-HCl was run through the column regardless of the extinction of the effluent, to ensure that the acid within the column was at a concentration of 0.03N.

When the washing of the column with 0.03N-HCl was complete, the 1.1 ml. of DNA hydrolysate was run into it. Then 50 ml. of 0.03N-HCl was passed through the column to elute the purinic acids. From this point on elution was carried out with 2N-HCl. A typical elution pattern is shown in Fig. 1. The adenine peak and the guanine peak were each collected in a weighed flask. The solvent coming off the column in between the adenine and guanine peaks was also collected. The run normally took slightly less than 3 hr. to complete.

The flasks containing the adenine and the guanine were weighed, and the actual weights of the adenine and guanine solutions calculated. Since the primary concern is to measure the A/G ratio rather than the absolute amounts of these compounds, it is a permissible simplification to assume that the weight in grams of each solution is equal to its volume in millilitres. If, however, it is desired to determine the absolute amounts of the two bases then the weight of

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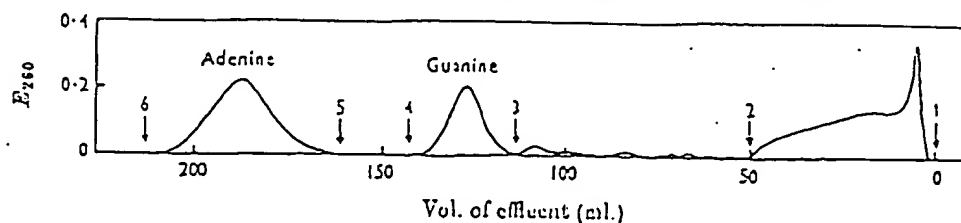


Fig. 1. Separation on Zeo-Karb 225 resin of adenine and guanine from DNA hydrolysed with 0.03N-HCl. The procedure is described in the Methods and Materials section. At arrow 1 elution was started with 0.03N-HCl; from arrow 2 onwards elution was carried out with 2N-HCl. The small peak eluted before guanine may be deoxycytidine. Effluent collected between arrow 3 and arrow 4 is the guanine fraction. Effluent collected between arrow 4 and arrow 5 is used as the blank. Effluent collected between arrow 5 and arrow 6 is the adenine fraction.

each fraction should be divided by 1.033 to give the volume in millilitres.

The extinction values of the adenine and guanine solutions were now read at suitable wavelengths in 5 cm. path-length cells in the Hitachi Perkin-Elmer spectrophotometer. The 2N-HCl coming off the column between the adenine and guanine peaks was used as the blank: this was to ensure that the effect of possible traces of ultraviolet-absorbing impurities from the resin that might contaminate the adenine and guanine fractions would be cancelled out by the fact that such traces would also be present in the blank. With adenine, measurements were made at 262, 275 and 310 m $\mu$ ; with guanine, measurements were made at 248, 265 and 310 m $\mu$ . In each case, the extinctions at the first two wavelengths were corrected for possible light-scattering effects by subtraction of the extinction at 310 m $\mu$ .

As a check on the purity of the fractions the calculated values of  $E_{262}/E_{275}$  for adenine, and of  $E_{248}/E_{265}$  for guanine, were compared with the values previously obtained with authentic samples of the two bases.

The concentrations of adenine and guanine were computed from the  $E_{262}$  and  $E_{248}$  values respectively by using the extinction coefficients already determined. The actual amounts of adenine and guanine were found by multiplying the concentration of each base by the volume of the solution. The A/G ratio was then calculated.

**Materials.** Adenine and guanine (both described as chromatographically pure) were obtained from Boehringer Corporation (London) Ltd., London, W. 5.

The 2.0N-HCl was made up by diluting 52.1 ml. (measured in a Gellenkamp 100 ml. graduated cylinder with a works certificate indicating the error) of constant-boiling HCl (20.24% w/w, HCl), obtained from British Drug Houses Ltd., Poole, Dorset, to 250 ml.

The polystyrenesulphonic acid resin used for the ion-exchange chromatography was Zeo-Karb 225 (over 200 mesh; 4.5% cross-linkage; code no. SRC12) manufactured by The Permutit Co. Ltd., London, W. 4. Before the column was made up, the resin was washed several times alternately with 2N-HCl and 2N-NaOH, and finally with 0.03N-HCl. The resin occupies a larger volume when suspended in 0.03N-HCl than when suspended in 2N-HCl: hence the necessity to resuspend in 0.03N-HCl before making the 17 cm. column.

The DNA used in this work was calf thymus DNA (sodium salt; type I; highly polymerized, lot 10433-0960) from the Sigma (London) Chemical Co. Ltd., London, S.W. 6.

## RESULTS

Twelve analyses of calf thymus DNA were carried out as described in the Methods and Materials section. The mean value of  $E_{262}/E_{275}$  for the adenine fractions was 1.601, which is within 1% of the value obtained with authentic adenine. The mean value of  $E_{248}/E_{265}$  for the guanine fractions was 1.543, which is also within 1% of the value obtained with an authentic sample of the base.

The 12 values obtained for the A/G ratio are listed in Table 1, together with the values of GC content calculated from the A/G ratios. The standard deviation of the A/G ratios obtained from these data is 0.011. With the old method (Kirk, 1963) the standard deviation of the A/G ratios was 0.03: thus the modifications in the technique have reduced its intrinsic variability by about threefold. A standard deviation of 0.011 in A/G ratio corresponds to a standard deviation of 0.2% GC in the GC content, for a DNA with a base composition of about 45% GC. The standard deviation of the A/G ratios tells us how much variability there is in this method arising out of the various random errors; it tells us nothing about the size of any systematic errors (i.e. errors having much the same effect on the A/G ratio in all determinations) that the method may have. Examples of possible systematic errors would be a significant non-linearity in the response of the spectrophotometer, or substantial contamination of one of the purine fractions by some other ultraviolet-absorbing component. At the moment there is no evidence for the presence of significant systematic errors in this technique: should such errors eventually be found then, being systematic, they would be relatively constant in their effects and so allowance could be made for them. Assuming, in the absence of evidence to the contrary, that there are no serious systematic errors present, then the standard deviations of 0.011 in the A/G ratio, and 0.2% in the GC content, may be taken as realistic estimates

Table 1. Values of the adenine/guanine ratio of calf thymus DNA

Expt. no.	A/G ratio	GC content calc. from A/G ratio
1	1.2322	44.80
2	1.1990	45.48
3	1.2050	45.33
4	1.2270	44.90
5	1.2094	45.25
6	1.2184	45.08
7	1.2322	44.80
8	1.2115	45.22
9	1.2161	45.12
10	1.2233	44.93
11	1.2274	44.90
12	1.2242	44.96
Mean = 1.2189		Mean = 45.07
= 1.219		= 45.1

Standard deviation of A/G ratio values = 0.011

∴ Standard deviation of GC content = 0.2% GC

of the absolute accuracy attainable with the method. It should be noted that the usefulness of the method is not impaired by any possible inaccuracies in the values for the extinction coefficients of adenine and guanine in 2N-hydrochloric acid. If other workers should obtain more accurate values for these constants, then any values for A/G ratios obtained with the present constants could simply be recalculated.

### DISCUSSION

The method that has been used more than any other for measurement of DNA base composition is the complete hydrolysis of the DNA with concentrated formic acid or perchloric acid and separation of the bases by paper chromatography (Vischer & Chargaff, 1948; Marshak & Vogel, 1951). An estimate of the standard deviation of GC content as determined by the formic acid method in expert hands may be obtained from the data on base composition of different kinds of mammalian DNA given in Table V of the review by Chargaff (1955). Ten different estimates are given of the standard error in the determination of the percentages of guanine and cytosine. The mean standard error in the determination of the percentage of guanine, calculated from these ten estimates, is 0.40 mole/100 moles; the corresponding value for cytosine is 0.28 mole/100 moles. The standard error (i.e. the estimate of the standard deviation) of the percentage of guanine plus cytosine is therefore  $\sqrt{[(0.40)^2 + (0.28)^2]}$ , which is 0.49.

The method of determination of DNA base composition that is now most commonly used is the measurement of buoyant density by equilibrium

density-gradient centrifugation in caesium chloride solution (Schildkraut, Marmur & Doty, 1962). Schildkraut *et al.* (1962) do not in fact give an estimate of the standard deviation of the GC contents derived by this method: however, they do say that 'in general, a value for the density seems to be good to  $\pm 0.001 \text{ g. cm.}^{-3}$ ', which is approx.  $\pm 1\%$  GC. A quantitative estimate of the inherent variability of this method may be obtained from the results of Tewari, Vötsch, Mahler & Mackler (1966), who obtained several values for the buoyant densities in caesium chloride of different kinds of yeast DNA. From their four values for the density of the whole-cell DNA, a standard deviation of  $0.0014 \text{ g. cm.}^{-3}$  may be calculated. From their 17 values for the density of a satellite band observed in DNA from mitochondria, a standard deviation of  $0.0015 \text{ g. cm.}^{-3}$  may be calculated. If we take the mean of these two values then, using the known relationship between buoyant density and GC content (Schildkraut *et al.* 1962), we can calculate that the standard deviation of the GC content values obtained by this method will be about 1.5% GC.

Base composition of DNA is often obtained by measuring the denaturation temperature,  $T_m$ , this being obtained by following the extinction at  $260 \text{ m}\mu$  as a function of temperature and noting the midpoint of the hyperchromic rise (Marmur & Doty, 1962). Marmur & Doty (1962) find the standard deviation of the  $T_m$  determinations for a given kind of DNA to be  $0.4^\circ$ . This means that the standard deviation of the GC content values obtained from estimates of  $T_m$  is 1.0% GC.

Information on base composition may be obtained by analysis of the spectra of native or denatured DNA. The simplest method of this type is that of Fredericq, Oth & Fontaine (1961), who derived an empirical relationship between the  $E_{240}/E_{260}$  ratio and the GC content. However, they give no indication of the standard deviation of the method.

Hirschman & Felsenfeld (1966) have developed methods based on measurements of extinction at four or more wavelengths on native or denatured DNA. From seven results obtained with denatured calf thymus DNA it is possible to calculate a standard deviation of 1.2% GC; for another seven results, obtained from the hyperchromic spectra (the difference between the spectra of fully denatured DNA and native DNA) of calf thymus DNA, the standard deviation is 1.4% GC.

On the basis of the data available in the literature, it therefore would seem that the inherent reproducibility of the method of DNA base analysis described in the present paper (which, for simplicity, I shall call the A/G-ratio method) is substantially better than that of the other methods in common

use. The standard deviation of the GC content obtained by this method is about 1.5%, which is 7.5 times the standard deviation of the GC content obtained by the other methods. However, by the use of the A/G-ratio method, a standard deviation of 1.5% GC is obtained, which is comparable to the standard deviation of 1.0% GC obtained by measurement of  $T_m$ . In the case of the A/G-ratio method, the standard deviation of 1.5% GC presents no difficulty in the preparation of plant tissue for the determination of base composition. The A/G-ratio method of base composition determination is to be used. The relative amount of DNA, instead of the amount of DNA required, is a function of the free base nucleotides. The A/G-ratio method of base composition determination is to be used.



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use. The standard deviations of values of GC content obtained by the usual chemical method (complete acid hydrolysis), determination of buoyant density, measurement of  $T_m$  and spectral analysis of whole DNA are, respectively, 2.5 times, 7.5 times, 5.0 times and 6.5 times the standard deviation of the A/G-ratio method. The main disadvantage of the present method is that for optimum accuracy it requires about 0.7 mg. of DNA. However, by using 10 cm.-path-length cells (these are obtainable for the Hitachi Perkin-Elmer spectrophotometer), it should be possible to obtain comparable accuracy with 0.35 mg. of DNA. The amount of DNA required could be decreased quite substantially before the accuracy fell to the levels obtained by determination of buoyant density, measurement of  $T_m$  or spectral analysis of whole DNA. In many cases, of course, the preparation of 0.7 mg. of DNA (or more, for replicate analyses) presents no problem: such amounts of DNA are readily prepared from whole bacteria or animal or plant tissue. However, in cases where it is difficult to prepare more than a few micrograms of a particular kind of DNA, the methods involving determination of buoyant density, measurement of  $T_m$  or spectral analysis of whole DNA would have to be used. When a knowledge of the nature and relative amounts of all the bases present in the DNA, instead of just the GC or AT content, is required, a method involving complete hydrolysis to the free bases with concentrated acid, or to the nucleotides with suitable enzymes, must be used. The A/G-ratio method therefore would be the method of choice in cases where an accurate

estimate of the overall base composition (as GC content or AT content) is required, and where perhaps as much as 2 mg. or more DNA (for three or more replicate determinations) can without too much difficulty be prepared.

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